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(57) Abstract

The present invention relates to a method for the diagnosis of symptomatic CMV disease, characterized in that the presence of mRNA encoding a late structural protein of the human Cytomegalovirus in a blood sample of an individual, suspected of carrying said disease, is detected, said method comprising the following steps: amplifying a target sequence within said mRNA using a primer pair capable f specifically reacting with said target sequence and suitable amplification reagents, reacting the sample, optionally containing amplified nucleic acid, with a labeled nucleic acid probe having a sequence complementary to part of the target sequence, detecting hybrids formed between the target sequence and the probe. The present invention further provides primers and probes for the amplification and detection f late pp67 HCMV mRNA.

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PRIMERS AND PROBES FOR THE AMPLIFICATION AND DETECTION OF CMV NUCLEIC ACID.

The present invention is concerned with oligonucleotides that can be used as primers and probes in the detection of human Cytomegalovirus (HCMV) mRNA. Furthermore a method for the diagnosis of HCMV disease is provided.

Human Cytomegalovirus is a ubiquitous Herpes-type virus, having a double stranded DNA genome of about 240,000 nucleotides in length that infects 40-80% of humans before puberty. A prominent feature common to all herpesviruses is their establishment of lifelong persistence after infection and their ability to cause recurrent infection after reactivation (Stevens, J.G.. Microbiol. Rev. 53, 318-332., 1989). HCMV also becomes latent after primary infection which often occurs without clinical symptoms. Even recurrent infection in most cases goes asymptomatic or leads to only mild disease in the immunocompetent host. However, in congenitally infected infants and immunocompromised patients, such as allograft recipients (Meyers, J.D., et al., J. Infect. Dis. 153, 478-488., 1986) or AIDS patients (Drew, W.L. J Infect. Dis 158, 449-456., 1988; Drew, W.L. Clin. Infect. Dis 14, 608-615., 1992), where the fine balance between the immune system and the latently existing virus is disturbed, HCMV may cause severe and sometimes life-threatening disease, including retinitis, gastrointestinal disorders, and encephalitis (Drew, 1992). Early administration of antiviral drugs like ganciclovir and foscarnet can have significant beneficial effects on the prognosis of a patient (for refs. see Jahn, G. et al., Intervirology 35, 60-72., 1993;Schmidt, G.M. et al., N. Engl. J Med. 324, 1005-1011., 1991). Therefore, with the availability of clinically effective antiviral therapy (Collaborative DHPG Treatment Study Group, 1986; Ringden, O., et al., Lancet 1, 1503-1506., 1985), early and sensitive diagnosis is of significant importance.

CMV specific antibodies, in particular IgM antibodies, can be used as a marker for CMV infection, but are of limited value when it comes to discrimination between latent and active infections. Most viral detection methods currently employed do not unambiguously allow for prediction of whether a given infection will be symptomatic. Furthermore serological methods are indirect and often lack sensitivity. Viral culture is a more direct diagnostic parameter for CMV viremia. Although CMV culture from blood cells appeared to be indicative for an active CMV infection, the method does not enable rapid diagnosis and is technically difficult. Moreover, viral culture does not necessarily correspond to HCMV disease. A reliable relation between virus isolation from peripheral leukocytes and the appearance of clinical symptoms may not exist in some immunosuppressed patients (Delgado, R. et al., J Clin. Microbiol. 30, 1876-1878., 1992). Also urinary or pharyngeal shedding of the virus frequently occurs without clinical symptoms and organ involvement. Amplification of HCMV DNA in peripheral leukocytes by polymerase chain reaction (PCR), although a very sensitive technique for CMV viremia, is not usable as a marker of clinically symptomatic HCMV infection either. Due to the high sensitivity of enzymatic amplification, occasionally HCMV DNA was detectable in peripheral leukocytes without HCMV-related disease. Latent viral genomes may be detected by this technique or a patient may remain HCMV-DNA positive over a prolonged period of time after the disease has resolved (for refs. see Jahn, G. et al., 1993;Zipeto, D., et al., J Clin. Microbiol. 30, 527-530., 1992; Delgado et al., 1992).

At the moment, the method of choice for the early diagnosis of acute symptomatic HCMV infection is the antigenemia assay based on immunological detection of the structural protein pp65 by using specific antibodies (St rch, G.A., et al., J. Clin. Microbiol. 32, 997-1003., 1994; Gerna, G., et al., J. Infect. Dis. 164, 488-498., 1991; Gerna, G., et al., J Clin. Microbiol. 30, 1232-1237.98., 1992). However, a matter of concern employing this method is its sensitivity. The number of pp65-positive cells in the early course of infection may be very low. Furthermore, in expressing cells stability of the pp65 antigen appeared to be limited (Chou, S., Curr. Opin. Infect. Dis. 5, 427-432., 1991) and sensitivity can be reduced due to the application of monoclonal antibodies rather than a pool of anti-pp65 antibodies that would recognize different epitopes of the protein.

Since viral replication requires transcription of mRNA species, the use of HCMV mRNA detection as a marker for active CMV infection was investigated (Bitsch, A. et al., J Infect. Dis 167, 740-743,, 1993; Velzing, J., et al. J Med. Virol. 42, 164-169., 1994).

Recently, HCMV infections were examined on the transcript level using RNA amplification (Bitsch, A. et al., J Infect. Dis 167, 740-743., 1993; Meyer, T.et al., Mol. Cell Probes. 8, 261-271., 1994; Gerna, G., et al., J Clin. Microbiol. 30, 1232-1237.98., 1993; Gerna, G., et al., J Clin. Microbiol. 30, 1232-1237.98., 1992; Velzing et al., 1994). In principle, like detection of viral antigens, analysis of viral transcripts expressed in association with viral replication should allow reliable diagnosis of symptomatic infections.

The presence of CMV mRNA is more likely to correlate with an active viral infection since earlier evaluations have shown that the presence of CMV mRNA does correlate with the presence of pp65 antigen (Velzing et al. Journal of Medical Virology 42:164-169, 1994).

The present invention provides a method for the detection of clinically symptomatic CMV disease based on the detection of late mRNA sequences of HCMV and nucleic acid sequences that could be used with said method.

Transcription of the late mRNA species requires viral replication and therefore could be specific for active infection. With the present invention it has been found that the detection of certain late mRNAs of HCMV is related to the appearance of clinical symptoms of HCMV disease. A method is provided for the diagnosis of HCMV disease characterized in that the presence of mRNA encoding a late structural protein of the human Cytomegalovirus in a blood sample of an individual suspected of carrying said disease is detected, said method comprising the following steps:

- amplifying a target sequence within said mRNA using a primer pair capable of specifically reacting with said target sequence and suitable amplification reagents,
- reacting the sample optionally containing amplified nucleic acid with a labeled nucleic acid probe having a sequence complementary to part of the target sequence and
- detecting hybrids formed between the target sequence and the probe.

When the presence or absence of certain late mRNAs in patients was correlated to their clinical status, a striking relation was observed between the presence of these late mRNAs and possibly CMV-related clinical symptoms.

The sensitivity and reliability of CMV mRNA detection is greatly dependent on primer selection, since there is sequence variation among strains of CMV in every region of the genome. Ideally, primer selection should be based on knowledge of interstrain

variability in candidate primer sequences and the consequences of mismatching at primer sites. (Chou S., Journal of Clinical Microbiology, 2307-2310, Sept.1992).
The need therefore exists for suitable oligonucleotides including nucleic acid sequences that can be used as primers and hybridization-probes for the amplification and subsequent detection of all strain variants of CMV.
The present invention is related to the detection of certain late HCMV mRNAs and provides suitable primers and probes for the amplification and subsequent detection of this mRNA. The binding sites of the primers and probes according to the present invention are located in the matrix tegument protein pp67 gene sequence, expressed during the late phase of CMV infection.
The term "oligonucleotide" as used herein refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides such as primers and probes.
The term "primer" as used herein refers to an oligonucleotide whether naturally occurring (e.g. as a restriction fragment) or produced synthetically, which is capable of acting as a point of initiation of synthesis of a primer extension product which is complementary to a nucleic acid strand (template or target sequence) when placed under suitable conditions (e.g. temperature and pH) in the presence of nucleotides and an agent for nucleic acid polymerization, such as DNA polymerase or reverse transcriptase. A primer must be sufficiently long to prime the synthesis of extension products in the presence of an agent for polymerase. A typical primer contains at least about 10 nucleotides in length of a sequence substantially complementary to the target sequence, but somewhat longer primers are preferred. Usually primers contain about 15-25 nucleotides but longer primers, up to 35 nucleotides may also be employed.
A primer will always contain a sequence substantially complementary to the target sequence, that is the specific sequence to be amplified, to which it may anneal. A primer may, optionally, also comprise a promoter sequence. With the term "promoter sequence" is meant a single strand (preferably the sense strand) of a nucleic acid sequence that is specifically recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription by which an RNA transcript is produced. In principle any promoter sequence may be employed for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Known and useful promoters are those that are recognized by certain bacteriophage polymerases such as bacteriophage T3, T7 or SP6.

Various techniques for amplifying nucleic acid are known in the art. One example of a technique for the amplification of a DNA target segment is the so-called "polymerase chain reaction" (PCR). With the PCR technique the copy number of a particular target segment is increased exponentially with a number of cycles. In each cycle a DNA primer is annealed to the 3' side of each strand of the double stranded DNA-target sequence. The primers are extended with a DNA polymerase in the presence of the various mononucleotides. The extension products are rendered single stranded by thermal denaturation and each strand can serve as a template for primer annealing and subsequent elongation in a following cycle. The PCR method has been described in Saiki et al.,Science 230, 135, 1985 and in European Patents no. EP 200362 and EP 201184.
Another technique for the amplification of nucleic acid is the so-called transcription based amplification system (TAS). TAS employs an RNA-transcript-production step from a DNA, synthesized to incorporate a segment with the target sequence and a

promoter, t enable transcription from the segment of a RNA with the sequence complementary t that of the target. Multiple cycles can be carried out as the RNA made in the transcription step can serve as template for making similarly transcribable DNA, which in turn, can be transcribed to yield additional RNA. The TAS method is described in International Patent Appl. no. WO 88/10315.
Yet another method for the amplification of nucleic acid is the nucleic acid sequence based amplification process ("NASBA") as described in European Patent no. EP 329822. Like TAS, NASBA includes a RNA-transcript production step using T7 RNA polymerase to transcribe multiple copies of RNA from a double stranded DNA template including a T7 promoter sequence.
An oligonucleotide sequence used as detection-probe may be labeled with a detectable moiety. Various labeling moieties are known in the art. Said moiety may, for example, either be a radioactive compound, a detectable enzyme (e.g. horse radish peroxidase (HRP)) or any other moiety capable of generating a detectable signal such as a colorimetric, fluorescent, chemiluminescent or electrochemiluminescent signal.

For the pp67 gene sequence interstrain variations between CMV AD169 and Towne exist. Therefore, for this target two primer pairs were chosen, CMV-pp67-1 and CMV-pp67-4 (Table 1), either of which was derived from the same region of the gene but each based on a different laboratory strain. An example of oligonucleotides for the detection of pp67 mRNA are oligonucleotides, 10-35 nucleotides in length comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group consisting of:
5'-GGGTCGATTCAGACTGA-3'
5'-GGGTCGATTCGAGACCGA-3' and
5'-GACCTGATATCCCTCCATATA-3'.

A preferred primer set according to the present invention includes the following oligonucleotide sequences:
5'-aattctaatacgactcactatagggagaGGGTCGATTCAGACTGA-3' or
5'-aattctaatacgactcactatagggagaGGGTCGATTCGAGACCGA-3' in combination with 5'-GACCTGATATCCCTCCATATA-3'.
The T7 promoter sequence is shown in lower case, but may be replaced by any other suitable promoter sequence.
A probe that may be used for the detection of the amplificate generated using this primer set may comprise an oligonucleotide consisting essentially of the following sequence: 5'-GGATTCGGACTTTCCGTTCGA-3'
Probes comprising said sequence are also part of the present invention.

For RNA amplification (as with the method according to the invention), the NASBA technology, or another transcription based amplification technique, is a preferred technology. This amplification method consists of an in vitro primer-directed amplification of a specific RNA region (Kievits et al., 1991).
If RT-PCR is used for the detection of viral transcripts differentiation of mRNA- and DNA-derived PCR products is necessary. For spliced transcripts, like the IEA mRNA, the exon-intron structure can be used (Velzing et al., 1994). However, mRNA species encoding the late structural proteins are almost exclusively encoded by unspliced transcripts. DNAse treatment prior to RT-PCR can be employed (Bitsch, A. et al., J

Infect. Dis 167, 740-743., 1993; Meyer, T.et al., Mol. Cell Probes. 8, 261-271., 1994), but sometimes fails to remove contaminating DNA sufficiently (Bitsch, A. et al., J Infect. Dis 167, 740-743., 1993).
In contrast to RT-PCR, NASBA, which is based on RNA transcription by T7 RNA polymerase (Kievits et al., 1991), does not need differentiation between RNA- and DNA-derived amplification products since it only uses RNA as its principal target. NASBA enables specific amplification of RNA targets even in a background of DNA. Especially for unspliced targets like almost all late HCMV gene transcripts, this method is beneficial as it circumvents DNAse treatment which occasionally might be inefficient (Bitsch, A. et al., J Infect. Dis 167, 740-743., 1993).
This method was used for the analysis of CMV transcripts in whole blood samples from transplant recipients and HIV-infected individuals.

Test kits for the detection of CMV in clinical samples are also part of the present invention. A testkit according to the invention may comprise a set of primers according to the invention and a probe according to the invention. Such a test kit may additionally comprise suitable amplification reagents such as DNA and or RNA polymerases and mononucleotides. Test kits that can be used with the method according to the invention may comprise the primers and probe according to the invention for the amplification and subsequent detection of pp67 mRNA.

The invention is further exemplified by the following example.

EXAMPLES:

Example 1: Analysis of CMV DNA and mRNA in clinical samples.

MATERIALS AND METHODS

Clinical Specimen
Samples from patients clinically at risk of infection with CMV were analyzed for the presence of mRNAs encoding the immediate early antigen (IEA) or the matrix tegument protein pp67 expressed during the late phase of CMV infection.
The thirty-five blood samples were obtained from immunocompromised patients including 22 heart, liver, or kidney transplant recipients, 8 AIDS patients, two patients with leukemia, one patient with a myelodysplastic syndrome, one patient with primary Epstein Barr virus mononucleosis, and one patient with Kaposi sarcoma. Ethylene diaminotetraacetic acid (EDTA) anticoagulated blood samples submitted consecutively as received by the laboratory of the University Hospital Rotterdam Dijkzigt, Rotterdam, The Netherlands, were mixed with nine volumes of Lysis buffer [50 mM Tris-Hydrochloric acid (pH 6.4); 20 mM EDTA; 1.3% (w/v) Triton X-100; 5.25 M Guanidinium thiocyanate] and stored at -70°C until use.

Nucleic acid isolation

From the anticoagulance-treated blood specimens total nucleic acid was isolated using guanidinium thiocyanate-mediated cell lysis and adsorpti n of nucleic acid to silica particles (Boom et al. Journal of Clinical Micr biology 28, 495-503, 1990).
Whole blood samples in Lysis buffer were thawed and from each sample 1 ml (equivalent to 100 µl whole blood) was transferred into an Eppendorf tube. Subsequently, 70 µl of Hydrochloric acid-activated silicum dioxide particles [size-selected suspension of 1 mg/ml in 0.1 M Hydrochloric acid (Sigma); see ref. Boom et al., 1990] were added and the suspension was incubated during 10 minutes at room temperature with regular vortexing. Nucleic acid bound to the silica was spun down by centrifugation. Pelleted silica particles were washed twice with 1 ml GuSCN wash buffer [50 mM Tris-Hydrochloric acid (pH 6.4); 5.25 M Guanidinium thiocyanate], followed by two washing steps with 1 ml 70% ethanol and a single washing step with 1 ml acetone. After each washing step, the suspension was briefly centrifuged and the silica pellet was resuspended in the next washing solution by thorough mixing. After removal of the acetone, the silica particles were dried by incubation at 56°C in a heating block during 10 minutes. Nucleic acid was eluted from the silica particles by incubation in 100 µl distilled water at 56°C during 10 minutes. Finally, the silica particles were spun down again and the supernatant was carefully pipetted into fresh reaction tubes avoiding any carry-over of silica. Extracted nucleic acid samples were stored at -70°C until use.

Prior to the detection of CMV mRNAs in these isolates, the integrity and amount f extracted RNA was validated.
Samples were analyzed for the presence of U1 snRNP-specific A protein (U1A) mRNA (Sillekens, P.T.G., et al., EMBO J. 6, 3841-3848., 1987), being a relatively low abundant message, transcribed from a cellular housekeeping gene. As revealed by Northern blot analysis, presence of amplifiable U1A mRNA was obvious in all samples (data not shown).

Primers and probes

Sequences and polarity of the primers and of the probes used for specific detection, are shown in Table 1.
All oligonucleotide primers and probes were synthesized on a PCR-MATE 391 DNA synthesizer (Applied Biosystems) using phosphoramidite biochemistry.
Oligonucleotides for ELGA detection (see below) were synthesized with a 5'-amino link (Aminolink 2; Applied Biosystems) for subsequent coupling of Horse Radish Peroxidase (HRP).
Amplification primers were purified by electrophoretically separating the crude oligonucleotide solutions over a 20% polyacrylamide/7M Urea slabgel and subsequent elution of the full-length oligonucleotide from the corresponding gel band. After elution from the gel slices and concentration by ethanol precipitation, primers were dissolved in Milli-Q water and concentrations determined by OD(260 nm) measurement.
Detection probes were conjugated with HRP (Boehringer) by coupling the enzyme to the amino link of the oligonucleotide using the cross-linking reagents SDPD (Pharmacia) and EMCS (Fluka). Unbound HRP was removed ver a Qiagen Tip-100 column (Qiagen). The HRP-labeled oligonucleotides were purified by polyacrylamide gel electrophoresis and subsequent elution of the HRP-oligonucleotides from the gel slices by vernight incubation in water. The amount of HRP-conjugated

oligonucleotide was calculated from OD(260nm) and OD(400 nm) measurement. The solutions were stored at -70°C.

NASBA amplification

Amplifications were performed using NASBA, since this amplification technology is capable of specifically amplifying RNA in a background of DNA. These amplification reactions were carried out using a standard NASBA protocol:

To set up an amplification reaction, 10µl of 2.5 x reaction buffer [100mM Tris-Hydrochloric acid (pH 8.5); 30mM Magnesium chloride; 105mM Potassium chloride; 12.5mM Dithriothreitol; 2.5 mM of each of dNTP; 5mM of ATP, CTP and UTP; 3.75mM of GTP; 1.25mM of ITP] was added to a reaction tube together with 6.25µl 4 x primermix [0.8µM of each primer; 60% Dimethylsulphoxide], 5µl nucleic acid solution, and 1.75 µl distilled water. This mixture was heated at 65 °C during 5 minutes, after which the tubes were placed at 41°C. Two µl of enzyme mix [40 units T7 RNA polymerase; 8 units AMV reverse transcriptase; 0.1 unit RNase H; 1.25 µg/µl BSA] were added and the contents of the tube was mixed by gentle tapping. The reaction was incubated at 41 °C for 90 minutes and stopped by placing it at -20°C.

Polymerase Chain Reaction

For the detection of the corresponding genes of the HCMV mRNAs PCR amplification was used (Performed as described in Saiki et al.,Science 230, 135, 1985).

As template DNA, 5 µl nucleic acid solution were added to a total of 20 µl of reaction mixture for amplification containing the appropriate primer pair (15 pmol each), deoxyribonucleoside triphosphates (200 µM each; Pharmacia), 2 µl of 10 x PCR buffer (Perkin-Elmer), and 1.25 units Taq polymerase. Reactions were overlaid with 100 µl of mineral oil to prevent evaporation. The amplification was performed in a DNA thermal cycler (Perkin-Elmer) by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, chain extension at 72°C for 2 min, and a final extension segment at 72°C for 10 min.

Southern blot analysis of PCR-amplified products.

Amplified DNA was transferred from a 2.0 % pronarose gel (Hispanagar, S.A.) to a nylon membrane (Zeta-probe; BioRad, USA) by vacuum blotting in 2 x SSC [1 x SSC is 150 mM Sodium chloride; 15 mM Sodium citrate] during 2 hours. Membranes were preincubated at 50°C in a hybridization solution [0.5 M Sodium phosphate (pH 7.2); 7% Sodium dodecyl sulphate] during 30 minutes prior to the addition of 32P labeled oligonucleotide probe to a final concentration of about 105 cpm/ml. Hybridization was performed overnight at 50oC. Subsequent washings were carried out at 50oC in 0.3 x SSC supplemented with 0.1 % SDS. Autoradiography was performed for several hours at -70oC with Kodak Royal X-omat film and intensifying screens.

Analysis of NASBA-amplified products (ELGA)

For the analysis of NASBA products a non-radio-active enzyme linked gel assay (ELGA) based on liquid hybridization was used.

Hybridization of amplification product to a specific HRP labeled oligonucleotide probe was perf rmed by mixing 2µl of a amplification reaction with 1 µl 5 x SSC, 1µl concentrated loading buffer [25% (v/v) Glycerol; 10mM Sodium phosphate buffer (pH

7.0); 0.05% bromophenol blue; 0.01% Xylene cyanol], and 1µl HRP-labeled oligonucleotide solution containing about 1010 molecules per µl, followed by incubation at 45oC during 15 minutes. After hybridization, half of the reaction mixture was directly applied onto a 7% polyacrylamide gel supplemented with 0.04% (w/v) dextrane sulphate. After separation of bound and unbound HRP-labeled oligonucleotide by electrophoresis, the probe was visualized in the gel by direct staining with 50 ml substrate solution [125µg 3,3',5,5'-tetramethylbenzidine per ml; 0.003 % Hydrogen peroxide; 100mM Sodium citrate buffer (pH 5.2)] for about 10 minutes at room temperature. Finally, the gel was fixed by overnight incubation in a 50% (v/v) methanol solution and air dried.

RESULTS:

Primers and sensitivity

To determine the analytical sensitivity attainable in NASBA with the primer pairs a standard dilution series of in vitro generated CMV RNA was evaluated. An RNA template of known concentration was generated in vitro from a cloned subfragment of the CMV AD 169 EcoRI fragment J (Schrier, R.D., et al., Science 230, 1048-1051., 1985) for IEA and a CMV clone encompassing the pp67 gene for pp67. Standard dilution series were prepared from the in vitro generated RNA.

Sensitivity of the CMV-IEA (E4) primer set is shown in figure 1 and was reproducibly found to be at least 100 molecules of in vitro generated RNA input in NASBA reaction. For the CMV-IEA(E2/E3) primer pair a comparable sensitivity of 100 molecules could be achieved.

Primer performance was evaluated on in vitro generated RNA transcribed from a cloned fragment of the pp67 gene of CMV AD169. For the CMV-pp67-4 primer pair, derived from AD169, NASBA conditions could be optimized such that the sensitivity of this primer pair was 100 molecules of in vitro generated RNA. As could be anticipated from the mismatches of the downstream primer of CMV-pp67-1, based on the Towne strain sequence, no amplification product could be generated with this primer pair from the CMV AD169-derived pp67 RNA.

To establish whether in addition to in vitro generated RNA also the genuine viral mRNAs could be identified by these primer sets, total nucleic acid from fibroblast cells infected with CMV AD 169 was extracted and amplified by NASBA. All primer sets revealed hybridization signals on Northern blot that correspond to CMV specific RNA derived amplification products. Northern blot analysis was performed as described below.

Based on ten-fold dilution series of the recombinant plasmids used for in vitro transcription of IEA RNA or pp67 RNA, the lower detection limit of the NASBA primer pairs, when used in PCR, was about 50-100 genome equivalents.

Detection of HCMV mRNA and DNA.

Because the internal U1A mRNA control was positive in all samples, the entire series of 35 specimens was further analyzed for the presence of the IEA gene and its corresponding mRNA. When amplification by PCR was performed, eighteen samples were positive for CMV-DNA when using the IEA gene primers (table 2). However,

PCR amplification with the pp67 gene primers failed to detect CMV-DNA in two of these eighteen samples (OT28 and OT34, Table 2). This indicates that despite the fact that already two primer pairs were used for the pp67 gene, still two samples were falsely negative for CMV DNA. Most likely, this is due to sequence variation among the clinical strains in this part of the HCMV genome, since the sensitivity of the primer sets for the IEA gene target and the pp67 gene target are comparable.

When the samples were analyzed for IEA mRNA by NASBA amplification with the same primer pairs as used for DNA detection by PCR, essentially all samples that were positive by PCR were also found positive by NASBA. Therefore with the exception of a single patient sample in all specimens positive for CMV-IEA DNA the cognate mRNA could be detected as well.

Analysis for pp67 mRNA by NASBA revealed a strikingly different result.

In contrast to IEA for which, with the exception of a single sample, all DNA-positive samples were positive for IEA-mRNA as well, pp67 mRNA could only be detected in a subset of the CMV-DNA positive patients (Table 2). In Table 3, the data for mRNA and DNA detection for the IEA target and the pp67 target are summarized. When the presence or absence of pp67 mRNA in these patients was correlated to their clinical status, a striking relation was observed between the presence of pp67 mRNA and possibly CMV-related clinical symptoms (Table 4).Two patients in this group showed symptoms of transplant rejection with graft dysfunction and fever. Clinical diagnoses in other patients were gastritis after heart transplantation and retinitis in a kidney transplant recipient. Retinitis was further observed in three CMV-pp67 mRNA positive AIDS patients, two of which also suffered from esophagitis.

Table 1: Primers and probes for PCR and NASBA amplifciation of CMV targets

Primer pair	Oligonucleotides	Sequence	CMV target
CMV-IEA (E4)	CMV-IEA 1.1	5'-aattctaatacgactcactatagggagaCTTGCTCACATCATGCAGCT-3'	IEA-exon 4
	CMV-IEA 1.2	5'-aattctaatacgactcactatagggagaCTTGGTCACATTATAGAGTT-3'	
	CMV-IEA 2.1	5'-TGAGCCTTTCGAGGAGATGAA-3'	
	CMV-IEA HRP-1	5'-AGGATAAGCGGGAGATGTGGAT-3'	
CMV-pp67-1	CMV-pp67 1.1	5'-aattctaatacgactcactatagggagaGGGTCGATTCGAGACCGA-3'	pp67
	CMV-pp67 2.1	5'-GACCTGATATCCCTCCATATA-3'	
	CMV-pp67 HPR-1	5'-GGATTCGGACTTTCCGTTCGA-3'	
CMV-pp67-4	CMV-pp67 1.2	5'-aattctaatacgactcactatagggagaGGGTCGATTC-AGACTGA-3'	pp67
	CMV-pp67 2.1	5'-GACCTGATATCCCTCCATATA-3'	
	CMV-pp67 HRP-1	5'-GGATTCGGACTTTCCGTTCGA-3'	

T7 promoter sequence in downstream primers is given in small characters.

Table 2: Analysis of CMV-IEA and CMV-pp67 DNA and mRNA in clinical specimens

No.	IEA-DNA (PCR)	IEA-mRNA (NASBA)	pp67-DNA (PCR)	pp67-mRNA (NASBA)
0T01	-	-	-	-
0T02	++	++	++	++
0T03	-	-	-	-
0T04	++	(+)	++	++
0T06	-	-	-	-
0T07	+	+	(+)	-
0T09	+	++	(+)	-
0T10	-	-	-	-
0T11	-	-	-	-
0T12	++	++	++	(+)
0T13	++	++	++	(+)
0T14	++	++	++	++
0T16	-	-	-	-
0T17	++	++	++	(+)
0T18	-	-	-	-
0T19	++	++	(+)	-
0T20	-	-	-	-
0T21	-	-	-	-
0T22	++	++	++	++
0T23	-	-	-	-
0T24	++	++	++	++
0T26	++	++	+	-
0T27	-	-	-	-
0T28	(+)	++	-	-
0T29	++	++	++	-
0T31	++	++	++	++
0T32	-	-	-	-
0T33	++	+	++	++
0T34	(+)	-	-	-
0T35	-	-	-	-
0T36	-	-	-	-
0T37	-	-	-	-
0T38	-	-	-	-
0T39	-	-	-	-
0T40	(+)	++	(+)	-

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No. patient sample number

++ strongly positive

\+ positive

(+) weakly positive

Table 3

CMV target	DNA-pos/RNA-pos	DNA-pos/RNA-neg	DNA-neg/RNA-neg
IEA (n = 35)	17	1	17
pp67 (n= 35)	9	7	19

- Two samples negative for pp67 DNA and mRNA while positive for IEA DNA and mRNA.
- A number of CMV-positive samples does not contain detectable levels of pp67 mRNA.

Table 4: Correlation between presence of HCMV pp67 mRNA and clinical status.

A. pp67 DNA-pos/RNA-pos patients

Patient	Clinical status
Heart transplantation	graft rejection, fever, bronchial carcinoma, immunocytoma
Kidney transplantation	retinitis, fever
Kidney transplantation	graf rejection, fever
Kidney transplantation	
AIDS	retinitis, lobular hepatitis
AIDS	retinitis, esophagitis
AIDS	retinits, esophagitis
Heart transplantation	gastritis
AIDS	epilepsy

B. pp67 DNA-pos/RNA-neg patients

Patient	Clinical status
Kaposi sarcoma	cryptococcus meningitis
AIDS	
Kidney transplantation	
Kidney transplantation	
Heart transplantation	
Heart transplantation	secundary EBV infection, immunocytoma
Heart transplantatin	

C. pp67 DNA-neg/RNA-neg patients

Patient	Clinical status
Leukemia	
Liver transplantation	elevated liver enzymes
Heart transplantation	graft rejection
Heart transplantation	graft rejection
Heart transplantation	
AIDS	lobular pneumonia, candida infection
–	acute myeloid leukemia, vasculitis
Heart transplantation	
–	primary EBV mononucleosis
Kidney transplantation	
AIDS	
Heart transplantation	graft rejection
AIDS	
Kidney transplantation	tuberculosis
–	myelodysplastic syndrome
Heart transplantation	seisure
Heart transplantation	sternum infection, staphylococcus aureas
Heart transplantation	
Heart transplantation	

5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Akzo Nobel N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM

(ii) TITLE OF INVENTION: Primers and probes for the detection of CMV nucleic acid.

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: EP 94202360.7
(B) FILING DATE: 18-AUG-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTCTAATA CGACTCACTA TAGGGAGACT TGCTCACATC ATGCAGCT 48

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCTAATA CGACTCACTA TAGGGAGACT TGGTCACATT ATAGAGTT 48

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TGAGCCTTTC GAGGAGATGA A 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGATAAGCG GGAGATGTGG AT 22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AATTCTAATA CGACTCACTA TAGGGAGAGG GTCGATTCGA GACCGA 46

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGGTCGATTC GAGACCGA 18

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GACCTGATAT CCCTCCATAT A 21

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGATTCGGAC TTTCCGTTCG A 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AATTCTAATA CGACTCACTA TAGGGAGAGG GTCGATTCAG ACTGA 45

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGGTCGATTC AGACTGA 17

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GACCTGATAT CCCTCCATAT A 21

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGATTCGGAC TTTCCGTTCG A 21

CLAIMS:

1. Method for the diagnosis of symptomatic CMV disease, characterized in that the presence of mRNA encoding a late structural protein of the human Cytomegalovirus in a blood sample of an individual, suspected of carrying said disease, is detected, said method comprising the following steps:
- amplifying a target sequence within said mRNA using a primer pair capable of specifically reacting with said target sequence and suitable amplification reagents,
- reacting the sample, optionally containing amplified nucleic acid, with a labeled nucleic acid probe having a sequence complementary to part of the target sequence
- detecting hybrids formed between the target sequence and the probe.

2. Method according to claim 1, wherein said mRNA is pp67 mRNA.

3. Method according to claim 1 or 2, wherein the mRNA is amplified, using a transcription based amplification technique.

4. Method according to claim 3, wherein said amplification technique is NASBA.

5. Oligonucleotide, corresponding to part of a nucleic acid sequence encoding HCMV pp67, said oligonucleotides being 10-35 nucleotides in length and comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group consisting of:
5'-GGGTCGATTCGAGACCGA-3'
5'-GGGTCGATTC-AGACTGA -3'
5'-GACCTGATATCCCTCCATATA-3', or its complementary sequence.

6. Oligonucleotide according to claim 5 linked to a promoter sequence.

7. Set of primers, for the amplification of a target sequence located within a HCMV pp67 sequence, comprising one primer consisting essentially of the nucleic acid sequence 5'-
aattctaatacgactcactatagggagaGGGTCGATTCAGACTGA-3'
linked to a promoter sequence and a second primer consisting essentially of the nucleic acid sequence 5'-GACCTGATATCCCTCCATATA-3'.

8. Set of primers, for the amplification of a target sequence located within HCMV pp67 sequence, comprising one primer consisting essentially of the nucleic acid sequence 5'-aattctaatacgactcactatagggagaGGGTCGATTC-AGACTGA-3'
linked to a promoter sequence and a second primer consisting essentially of the nucleic acid sequence 5'-GACCTGATATCCCTCCATATA-3'.

9. Use of any of the oligonucleotides according to claim 5 as a primer in the method of claim 2.

10. Use of an oligonucleotide consisting essentially of the sequence 5'-GGATTCGGACTTTCCGTTCGA-3', provided with a detectable label, as a probe in the method of claim 2.

11. Test kit for the diagnosis of HCMV disease comprising:
- one or more set(s) of primers according to claim 7, and /or 8,
- an oligonucleotide comprising a nucleic acid sequence substantially complementary to at least part of the amplified nucleic acid sequence defined by said set of primers, provided with a detectable label.

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(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SILLEKENS, Peter, Theodorus, Gerardus [NL/NL]; Boomstraat 3, NL-5291 ND Gemonde (NL). TIMMERMANS, Eveline, Catharina, Anna, Clasina [NL/NL]; De Elders 39, NL-5087 AM Diessen (NL).

(74) Agent: BEETZ, T.; Postbus 20, NL-5340 BH Oss (NL).

(81) Designated States: AU, CA, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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(54) Title: PRIMERS AND PROBES FOR THE AMPLIFICATION AND DETECTION OF CMV NUCLEIC ACID

(57) Abstract

The present invention relates to a method for the diagnosis of symptomatic CMV disease, characterized in that the presence of mRNA encoding a late structural protein of the human Cytomegalovirus in a blood sample of an individual, suspected of carrying said disease, is detected, said method comprising the following steps: amplifying a target sequence within said mRNA using a primer pair capable of specifically reacting with said target sequence and suitable amplification reagents, reacting the sample, optionally containing amplified nucleic acid, with a labeled nucleic acid probe having a sequence complementary to part of the target sequence, detecting hybrids formed between the target sequence and the probe. The present invention further provides primers and probes for the amplification and detection of late pp67 HCMV mRNA.

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3NSDOCID: <WO___9606191A3_I_>

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 95/03295

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 586 011 (EASTMAN KODAK CO) 9 March 1994 see page 7, line 16 - line 53 see claims	1,3,4
A	EP,A,0 329 822 (CANGENE CORP) 30 August 1989 cited in the application see the whole document	1-4
A	WO,A,93 13227 (CHIRON CORP) 8 July 1993 see SEQ ID 21	5

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 1996	15.02.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Andres, S

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/EP 95/03295

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MOL CELL PROBES, (1994 AUG) 8 (4) 261-71, MEYER, T. ET AL. 'Identification of active cyt megalovirus infection by analysis of immediate-early, early and late transcripts in peripheral blood cells of immunodeficient patients.' cited in the application see table 1 -----	1

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 95/03295

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0586011	09-03-94	JP-A-	7298897	14-11-95
EP-A-0329822	30-08-89	AT-T-	106948	15-06-94
		DE-D-	3850093	14-07-94
		DE-T-	3850093	03-11-94
		WO-A-	9102814	07-03-91
		ES-T-	2053648	01-08-94
		JP-A-	2005864	10-01-90
		US-A-	5409818	25-04-95
WO-A-9313227	08-07-93	EP-A-	0625214	23-11-94
		JP-T-	7502655	23-03-95
		US-A-	5407795	18-04-95